OFFICE OF NAVAL RESEARCH

ANNUAL PROGRESS REPORT

Report Prepared By: Albert E. Sobel, Ph. D.

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CONTRACTOR: Jewish Hospital of Brooklyn, Brooklyn 16, New York

PRINCIPAL INVESTIGATOR: Albert E. Sobel, Ph. D.

Assistants: Martin Burger
Albert Hanok
Joseph Zuckerman
Ali Gone, animal man
Jack Binkler, diener
Penni Ann Laurence, secretarial service

TITLE OF PROJECT: Studies of the Mechanism of Calcification with Special Reference to the Deposition and Removal of Cations Other Than Calcium and the Caries Problem.

Objectives: Solution of the caries problem and development of a method for the removal of poisonous trace metals from bones.

SUMMARY OF RESULTS:

1. The Local Factor of Calcification. That a complex of chondroitin sulfate with collagen may be responsible for the process of mineralization in bone is indicated from our studies during the past year. The steps that led to this development are given below.

The possible involvement of chondroitin sulfate in the calcifying mechanism was first proposed by us as an explanation of the reversible inactivation of calcification in vitro. It was further suggested from our present studies with toluidine blue and protamine which inactivates the calcifying mechanism. This inactivation is a function of the inactivator to calcium ratio. In the case of protamine and toluidine blue, unlike the previous inactivation with mineral salts, a more specific target, namely chondroitin sulfate, a mucopolysaccharide, can be postulated.

In attempting to relate the "local factor" to the state of polymerization of

chondroitin sulfate, the influence of calcium ions on metachromatic staining was investigated. With a constant amount of toluidine blue the degree of metachromasia increases with calcium ion concentration in solution, up to a maximum of about 15 ME/L. Above this concentration of calcium ions there is a gradual decrease in metachromasia. Prior shaking with calcium chloride increases the intensity of metachromatic staining in the ossifying matrix. In contrast to this, when chondroitin sulfate is extracted from bone, calcium ion competitively interferes with metachromatic staining. However, the metachromasia obtained with isolated chondroitin sulfate combined with isolated collagen responds to calcium ion in the same way as rachitic bone cartilage. When this complex was first shaken with calcium ions and then with phosphate a typical silver stain of in vitro calcification was obtained. Control specimens of collagen or chondroitin sulfate did not behave in this fashion.

The property of calcium ions increasing the degree of metachromasia seems to be typical of calcifying cartilage in the studies carried out to date. The appearance of metachromasia in bone cartilage did not correlate in all cases with calcifiability. However, when calcifiability was destroyed by various agents, metachromasia was not enhanced by calcium ions.

2. Composition of Bones and Teeth. Composition studies of bones and teeth in relation to blood and diet were extended for in vitro as well as in vivo calcification. The results can be expressed by the equation

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = K\left[\frac{CO_3}{PO_4}\right]_{solution} + b.$$

This may be seen in Figures 1, 2, and 3. For purposes of comparison the equations of distribution of two slightly soluble solids in relation to fluid composition are given below.

(a) $$\left[\frac{CO_3}{PO_4}\right]_{solid} = \frac{Sp\ CaHPO_4}{Sp\ CaCO_3}\left[\frac{(Ca^{++})(CO_3^{=})}{(Ca^{++})(HPO_4^{=})}\right]^N_{solution} = K\left[\frac{CO_3^{=}}{HPO_4^{=}}\right]^N_{solution}$$

(b) $$\left[\frac{CO_3}{PO_4}\right]_{solid} = \frac{Sp\ Ca_3(PO_4)_2}{Sp\ CaCO_3}\left[\frac{(Ca^{++})(CO_3^{=})}{(Ca^{++})^3(PO_4^{\equiv})^2}\right]^N_{sol'n} = K\left[\frac{(CO_3^{=})}{(Ca^{++})^3(PO_4^{\equiv})^2}\right]^N_{sol'n}$$

At present calculations are being made of the solubility product of $CaHPO_4$, $Ca_3(PO_4)_2$, and $CaCO_3$, at the ionic strength of blood serum and at the ionic strength of in vitro calcification to determine the correlation between the ideal case and our findings. In the in vivo experiments the serum phosphate level was regulated by the Ca:P ratio of the diet, the carbonate being constant. With high calcium (1.2%) - low phosphate (0.25%) diets the serum inorganic phosphate was low (approximately 3 mg.%), whereas with the low calcium (0.1%) - high phosphate (0.7%) diets serum phosphate was higher (approximately 9 mg.%). Intermediate dietary ratios of Ca:P produced intermediate serum phosphate levels.

3. Caries Susceptibility. These studies indicate definitely that composition is related to caries susceptibility. (See Table 1). That the rates of solution in acids of high carbonate enamel and dentin are greater is indicated in studies performed with high carbonate and low carbonate enamel and dentin. The data are given in Figures 4 and 5.

4. Mechanism of Lead Deposition and Removal. When one produces rickets with lead carbonate substituted for calcium carbonate in the diet such rachitic sections do not calcify in vitro as well as control sections. This implied that the local mechanism of calcification is either directly injured by lead or that the development of the local mechanism is retarded. These findings may explain the frequent occurrence of rickets in children with lead poisoning. A group of young Wistar rats were given 1% $PbCO_3$ in their diets for 30 days and were subsequently placed on diets devoid of lead which were high in calcium and low in phosphate or low in calcium and high in phosphate. Highlights of the findings are given in Tables 1-5 of the enclosed

technical report on lead deposition.

It is concluded that vitamin D during lead administration influences lead absorption and thus causes a rise in blood lead. In contrast tc this, after lead administration is ceased the main equilibrium is between blocd lead and bone lead. Here, vitamin D, to the degree that it causes a rise in serum phosphate, appears to produce a reciprocal drop in blood lead, as would be expected from the solubility product principle.

It is further concluded that the high calcium - low phosphate diet, by decreasing serum phosphate, causes a concomitant rise in blood lead at the expense of bone lead, while the high phosphate - low calcium diet causes a rise in serum phosphate which is paralleled by a low blood lead level and decreased loss of bone lead.

TABLE 1

INFLUENCE OF DIET ON CARIES SUSCEPTIBILITY IN THE COTTON RAT

DIET	TYPE OF TOOTH	NUMBER OF ANIMALS	NUMBER OF CARIOUS LESIONS	EXTENT OF CARIOUS LESIONS
High Ca) Low PO_4)	High CO_3	76	10.9	26.0
Low Ca) High PO_4)	Low CO_3	57	5.5	14.2
"P"			< 0.01	< 0.01

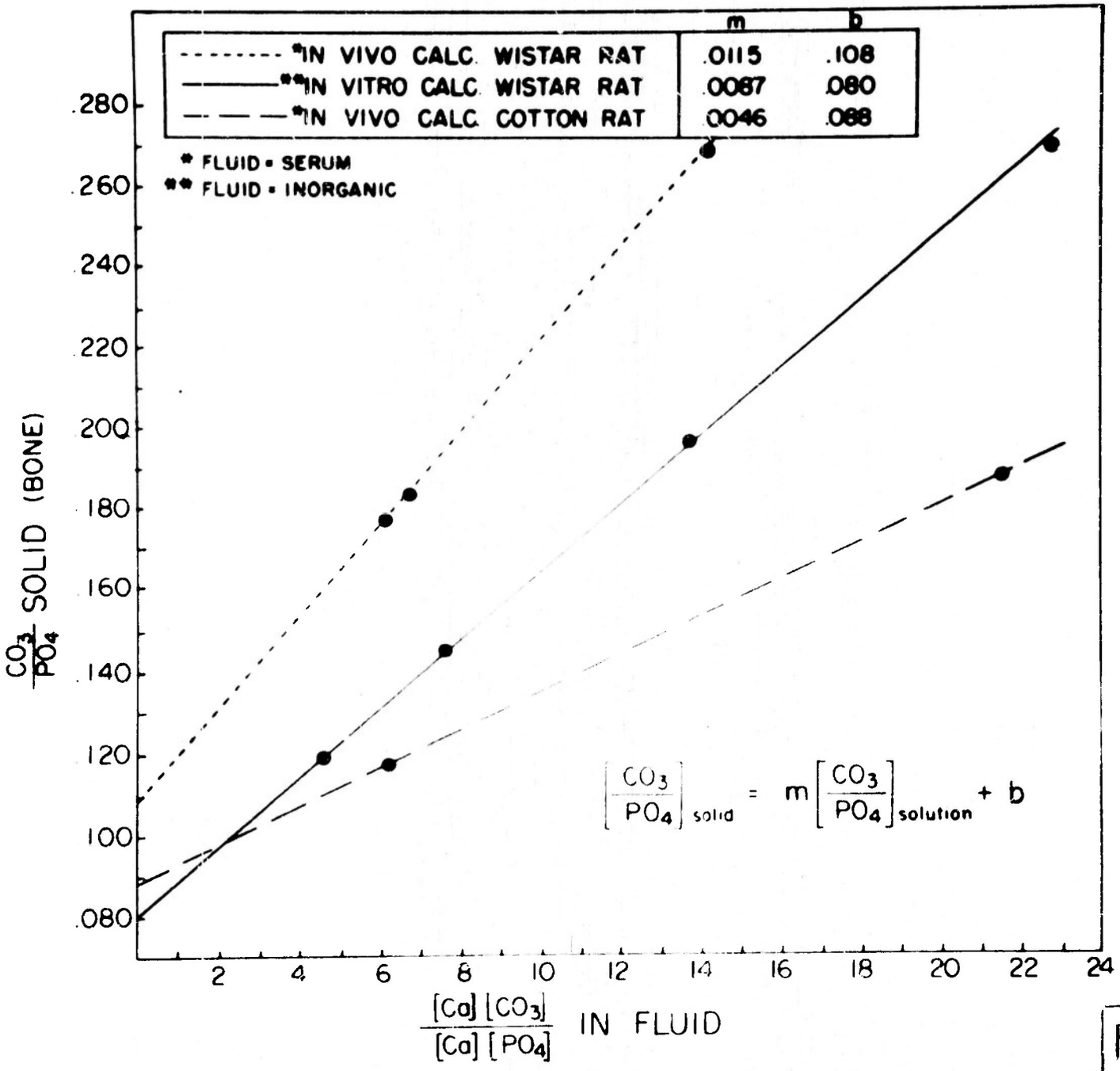
m
b
*IN VIVO CALC. WISTAR RAT .0115 .108
**IN VITRO CALC. WISTAR RAT .0087 .080
*IN VIVO CALC. COTTON RAT .0046 .088
* FLUID = SERUM
** FLUID = INORGANIC
.280
.260
.240
.220
.200
.180
.160
.140
.120
.100
.080
$\frac{CO_3}{PO_4}$ SOLID (BONE)
$\left[\frac{CO_3}{PO_4}\right]_{solid} = m\left[\frac{CO_3}{PO_4}\right]_{solution} + b$
2
4
6
8
10
12
14
16
18
20
22
24
$\frac{[Ca][CO_3]}{[Ca][PO_4]}$ IN FLUID

Fig. 1

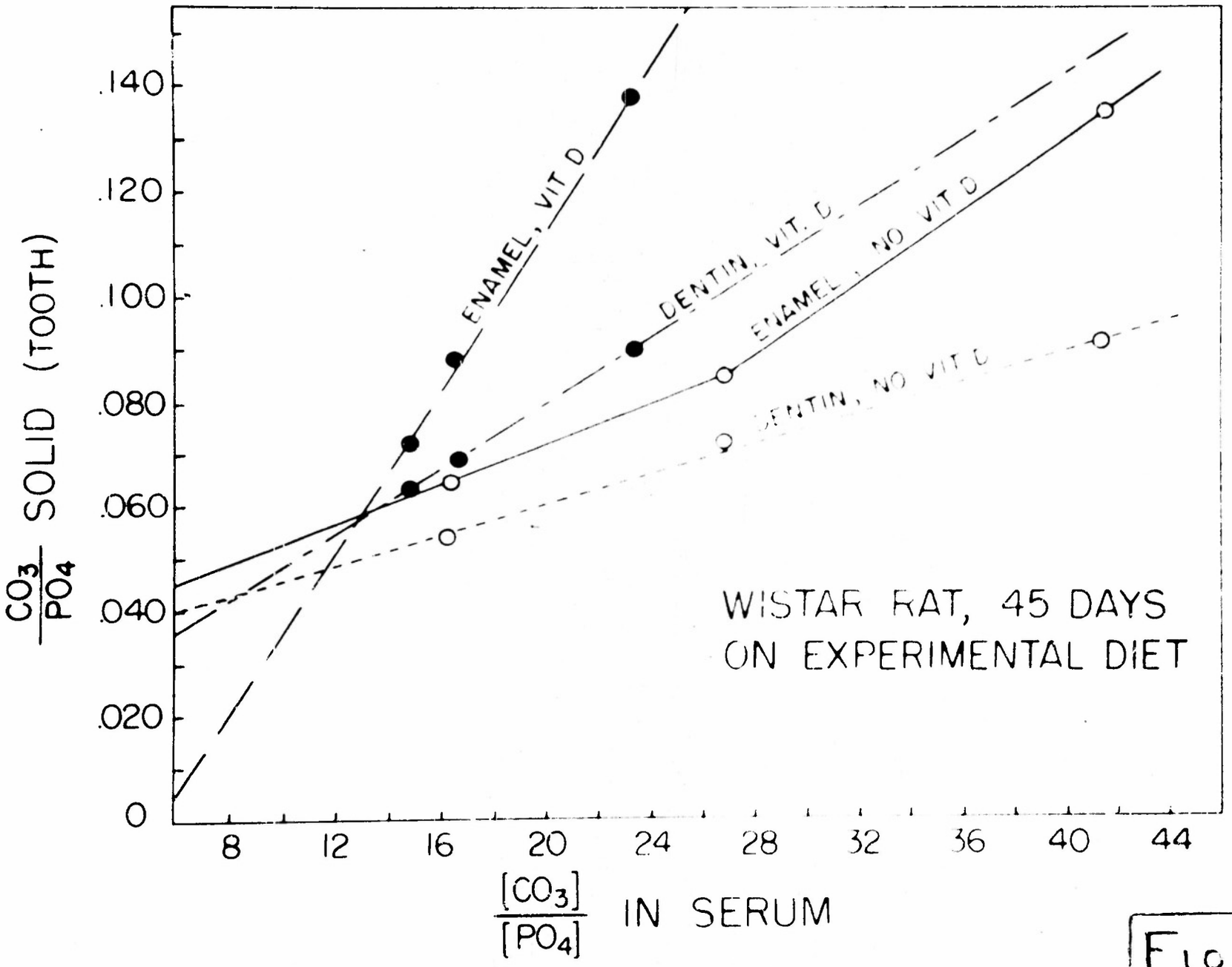
.140
.120
.100
.080
.060
.040
.020
0
$\frac{CO_3}{PO_4}$ SOLID (TOOTH)
ENAMEL, VIT D
DENTIN, VIT. D
ENAMEL, NO VIT D
DENTIN, NO VIT D
WISTAR RAT, 45 DAYS
ON EXPERIMENTAL DIET
8
12
16
20
24
28
32
36
40
44
$\frac{[CO_3]}{[PO_4]}$ IN SERUM

Fig. 2

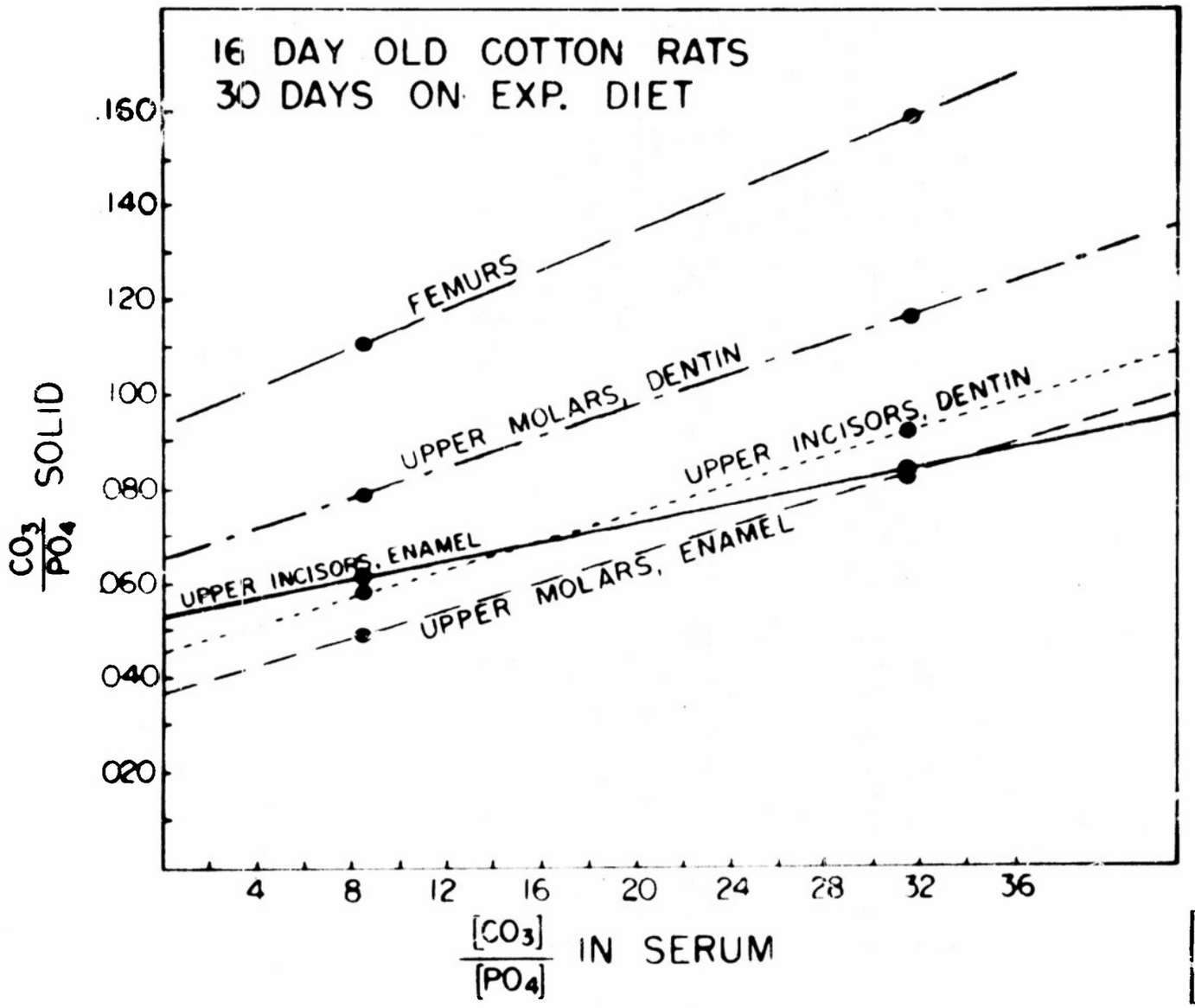
16 DAY OLD COTTON RATS
30 DAYS ON EXP. DIET
160
140
120
100
080
060
040
020
FEMURS
UPPER MOLARS, DENTIN
UPPER INCISORS, DENTIN
UPPER INCISORS, ENAMEL
UPPER MOLARS, ENAMEL
$\frac{CO_3}{PO_4}$ SOLID
4
8
12
16
20
24
28
32
36
$\frac{[CO_3]}{[PO_4]}$ IN SERUM

Fig. 3

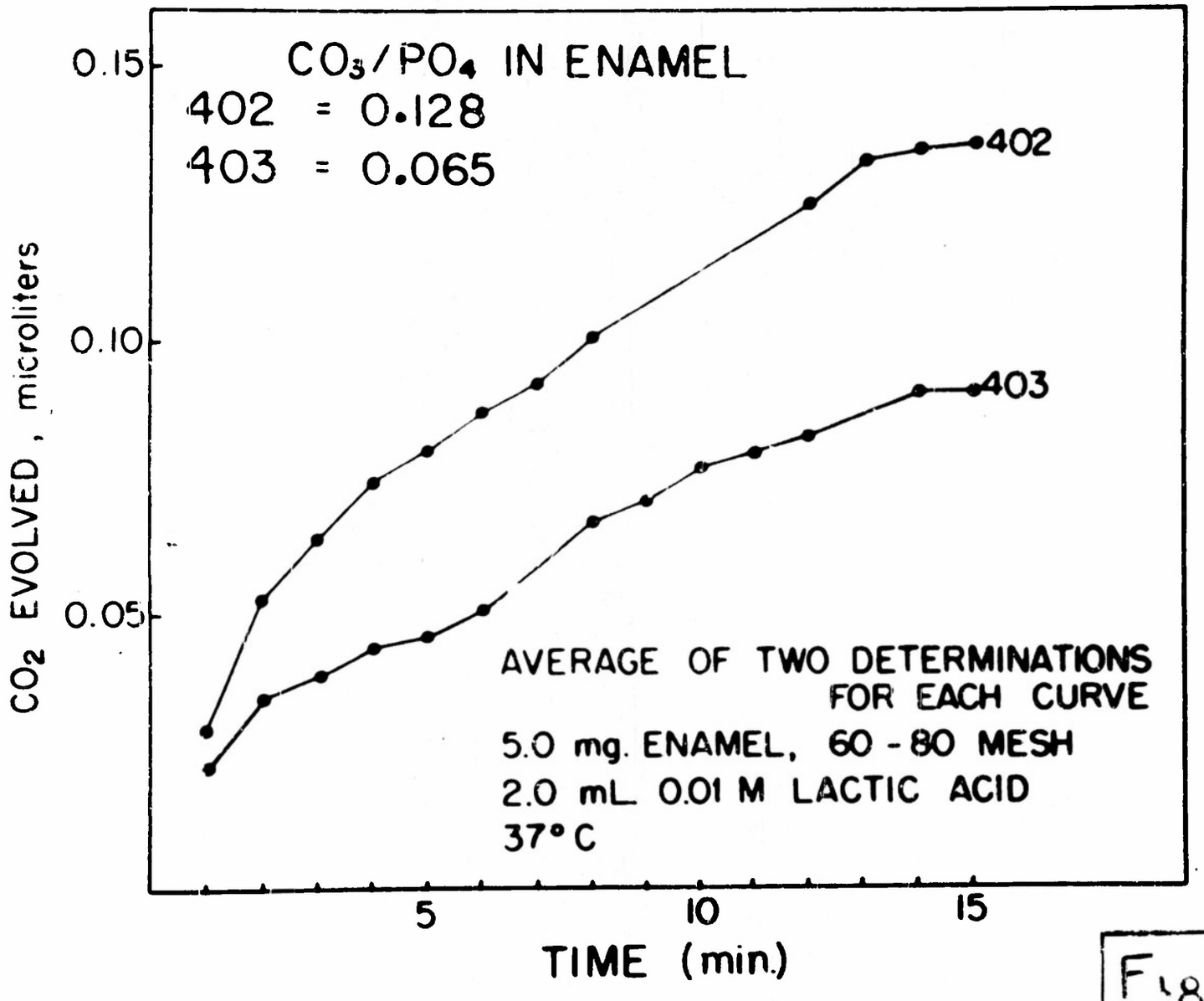
CO_3/PO_4 IN ENAMEL
402 = 0.128
403 = 0.065
402
403
AVERAGE OF TWO DETERMINATIONS FOR EACH CURVE
5.0 mg. ENAMEL, 60 - 80 MESH
2.0 mL 0.01 M LACTIC ACID
37° C
0.15
0.10
0.05
CO_2 EVOLVED, microliters
5
10
15
TIME (min.)

Fig. 4

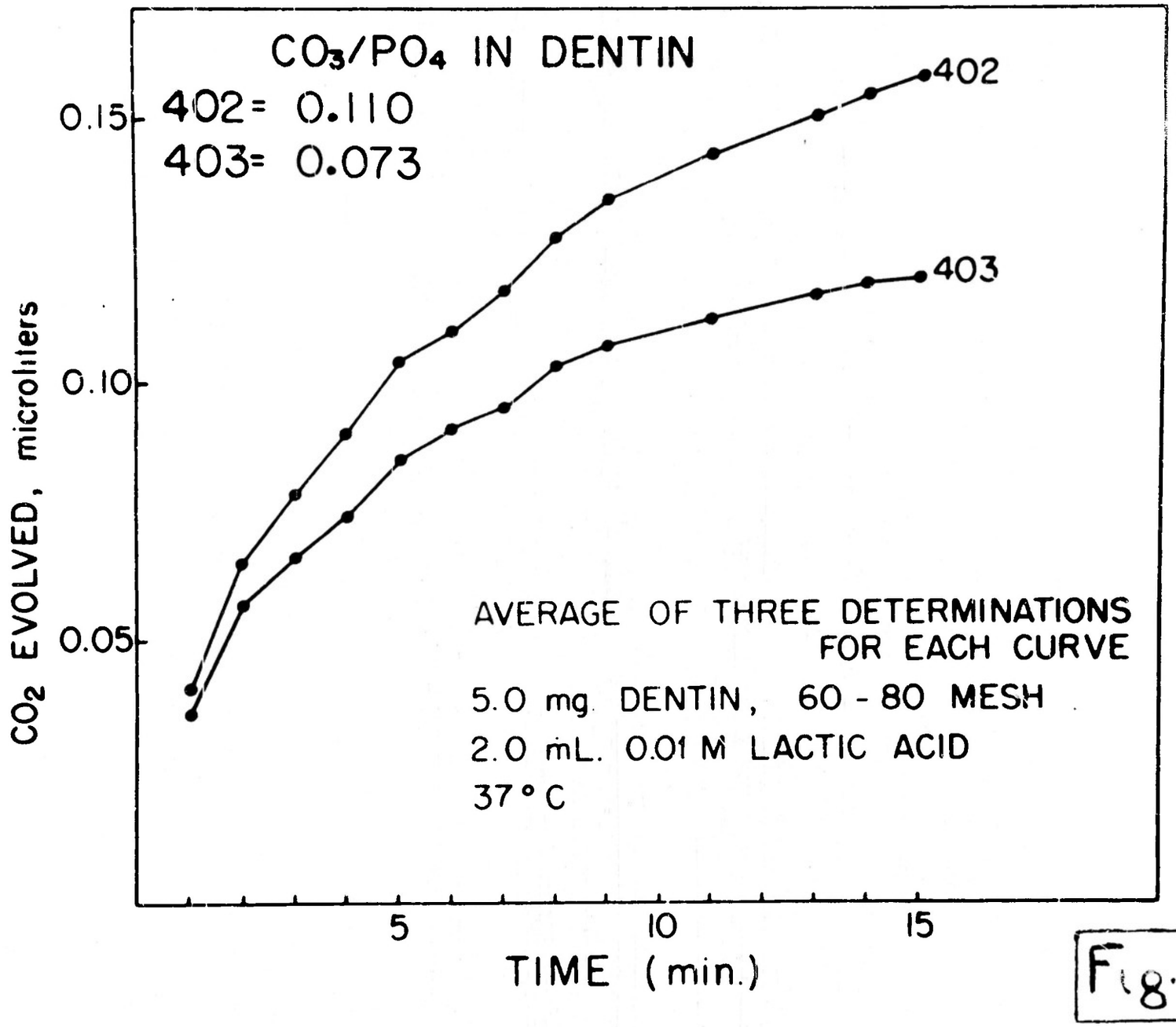
CO3/PO4 IN DENTIN
402= 0.110
403= 0.073
402
403
0.15
0.10
0.05
CO2 EVOLVED, microliters
AVERAGE OF THREE DETERMINATIONS FOR EACH CURVE
5.0 mg. DENTIN, 60 - 80 MESH
2.0 mL. 0.01 M LACTIC ACID
37° C
5
10
15
TIME (min.)

Fig. 5

PLANS FOR FUTURE

Immediate: Condensation of chondroitin sulfate prepared from various sources with collagen prepared from various sources by several methods. These collagen - chondroitin sulfate complexes will be examined by methods analogous to in vitro calcification to determine which of them may be responsible for the formation of bone-like minerals. If these experiments are successful an attempt will be made to condense keratin (the key protein of enamel) with chondroitin sulfate to determine whether these complexes may initiate the production of mineral similar in structure to that found in enamel. With all the above compounds the influence of calcium ions on the degree of metachromasia will be investigated, and if possible used as a guide to mineralization.

Our minimum hope is to learn more of the critical factors involved in the mechanism of calcification. Our maximum hope is to develop collagen - chondroitin sulfate and keratin - chondroitin sulfate complexes that may actually be useful in filling teeth and in prosthetic bone restoration.

Intensive study will be undertaken of factors that cause the deposition and removal of lead in bones and teeth, as a model to understand the deposition of cations other than calcium in bones and teeth. These studies will include the relation between composition of diet and blood, and removal and deposition of lead; the conditions under which lead deposits in vitro; and the influence of the presence of lead ions on calcification in vitro. Parallel metachromatic staining and enzyme studies are also projected.

Long range: The broad program is to understand the mechanism of calcification with special emphasis on the problem of caries and the removal of poisonous heavy metals from the body. The scope of this study was outlined in our initial suggestions in 1952. Details in addition to the immediate plans (above) are given below.

1. A thorough study of the inorganic models of bones and teeth will provide fundamental information on which we may draw in understanding teeth and formulating new ideas. The rate of formation of these compounds from solutions resembling the inorganic portion of intra- and extra-cellular fluid will be carefully studied for the same reason. At the beginning preference will be given to the various apatites (i.e., fluoro-, hydroxy-, and carbonate-apatite) that are known to exist in teeth or bones. Later the conditions that favor the production of apatites mixed with $CaHPO_4$, $CaCO_3$, and calcium citrate, will be investigated. Such mixtures appear to occur in teeth from the available evidence.

2. A careful investigation will be made of the physical state of the tooth enamel and dentin, as well as inorganic models of tooth and bone. The physical methods include X-ray diffraction, optical methods like refractive index and birefringence, and chemical analysis.

3. A careful study will be made of the influence of small amounts of fluoride on the composition of bones and teeth. Moreover, our experiments on in vitro calcification indicate such studies to be undertaken under the influence of low magnesium and high magnesium diets.

4. Caries-susceptibility in relation to composition of teeth in the cotton rat, and also the relationship that exists between tooth, blood, and bone composition, will be studied. We have already correlated the composition of tooth enamel and dentin and bone to that of blood and diet. The Cotton rat was chosen because it is susceptible to caries on diet 902.

5. Rates of solution and solubility of enamel and dentin of various compositions in weak acids and other solubilizing agents like citrates will be studied.

6. Extension of the composition studies will be made to include citrates, which have been reported to be present in teeth. The citrate content may be of special importance in view of the ready solubility of calcium citrates.

REPORTS AND PUBLICATIONS

1. The Chemistry of Bone and Tooth Formation, by Albert E. Sobel. Presented before a joint meeting of the Chicago Section of the American Chemical Society and the American Association of Clinical Chemists. Chicago, Illinois. May 22, 1953.

2. The Chemistry of Bone and Tooth Formation, by Albert E. Sobel. Presented before the House Staff of the Michael Reese Hospital, Chicago, Illinois. May 22, 1953.

3. The Local Factor of Calcification with Special Reference to Chondroitin Sulfate, by Albert E. Sobel and Martin Burger. Presented before the XIX International Physiological Congress, Montreal, Canada. September 2, 1953. Reprints enclosed.

4. The Chemistry of Bone and Tooth Formation, by Albert E. Sobel. American Chemical Society Tour No. 31. Talk delivered before local sections of the American Chemical Society at Olean, New York; Youngstown, Ohio; Columbus, Ohio; Wooster, Ohio; Painesville, Ohio; and Akron, Ohio. November 12, 13, 16, 17, 18, and 19, 1953.

5. The Chemistry of Bone and Tooth Formation, by Albert E. Sobel. Presented before the St. Louis Section of the American Chemical Society, St. Louis, Mo., December 7, 1953.

6. Local Factors in the Mechanism of Calcification, by Albert E. Sobel. Presented at Conference on Recent Advances in the Study of the Structure, Composition, and Growth of Mineralized Tissues, New York Academy of Sciences, New York, N. Y. January 23, 1954. Preprint enclosed.

7. Studies of Chondroitin Sulfate in Relation to the Mechanism of Calcification, by Albert E. Sobel and Martin Burger. To be presented before the Federation of American Societies of Experimental Biology, Atlantic City, New Jersey. April 11-16, 1954. Abstract enclosed.

8. Calcification XIV. Investigation of the Role of Chondroitin Sulfate in the Calcifying Mechanism, by Albert E. Sobel and Martin Burger. Submitted as technical report, NR 180 025. January 18, 1954.

9. The Biochemical Behavior of Lead II. The Influence of Calcium, Phosphorus, and Vitamin D on Lead in Blood and Bone After Withdrawal of Lead From the Diet, by Albert E. Sobel and Martin Burger. Submitted as technical report, NR 180 025. January 18, 1954.